Blood Transfusion Alters the Course and Outcome of *Plasmodium chabaudi* AS Infection in Mice

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The importance of severe anemia in the mortality of susceptible A/J mice during blood-stage *Plasmodium chabaudi* AS infection was assessed. Blood transfusion during and 2 to 3 days after peak parasitemia rescued 90% of susceptible mice from severe anemia and death and allowed these mice to clear the infection and acquire immunity to reinfection. However, blood transfusion prolonged the patency of the infection for up to 5 days after peak parasitemia. Blood transfusions in resistant C57BL/6 mice produced an identical effect, that is, prolongation of the patency of parasitemia. In addition, blood transfusion increased the numbers of gametocytes in both mouse strains. In both strains of mice, the rapid reduction in parasitemia, which occurs during crisis, was associated with the development of moderate levels of anemia. The possible mechanisms for the modulation of parasitemia by blood transfusion and the implications of the present observations for our understanding of the events which occur during crisis are discussed. It is proposed that parasitologic crisis is induced and/or maintained by physiological alterations associated with anemia.

Blood-stage *Plasmodium chabaudi* AS infection in mice follows either a lethal or a nonlethal course in a host strain-dependent fashion. Our laboratory has previously characterized the strain distribution pattern of resistance and susceptibility to *P. chabaudi* AS infection (18). Resistant C57BL/6 mice develop moderate levels of peak parasitemia, clear the infection to subpatent levels, and are subsequently immune to challenge infection. On the other hand, susceptible A/J mice experience higher levels of parasitemia and succumb to infection within 10 to 14 days postinfection. Susceptible mice suffer from severe anemia and shock prior to death. Severe anemia has been presumed to be the cause of death in susceptible mice, although this has not been formally proven (23). The experiments reported herein were initiated to assess the contribution of severe anemia to the mortality of susceptible A/J mice as a result of *P. chabaudi* AS infection. Our finding that blood transfusion rescues susceptible mice but prolongs the period of patent parasitemia and retards the occurrence of crisis forms the basis of this communication.

(This work was performed in partial fulfillment of the requirements for a Ph.D. by G.S.Y.)

MATERIALS AND METHODS

Parasite and experimental infections. C57BL/6 and A/J mice bred at the animal facility of the Montreal General Hospital were used at ages of 8 to 10 weeks. Mice were infected intraperitoneally with 10^6 parasitized erythrocytes (PRBC) prepared as previously described (23). Blood smears and RBC counts were obtained by using standard procedures as previously described (23).

Blood transfusion. Blood was obtained from noninfected donors (same strain and sex as recipients) by cardiac puncture with heparinized syringes. Pooled blood cells were washed twice and resuspended in saline. Blood transfusions were done by intraperitoneal injection of washed blood cells (0.7×10^9 to

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0.9×10^9 RBC in 1 ml of saline per mouse). Unless otherwise stated, transfusions were given beginning on the day of peak parasitemia and 2 to 3 days subsequently.

Histological and functional clearance studies. For histological studies, mice were injected with 0.2 ml of a dialyzed suspension of India ink and sacrificed 15 min later. Spleens and livers were weighed, fixed in buffered formalin, and processed for paraffin embedding. Sections (4 µm thick) were prepared and stained with hematoxylin and eosin. For clearance studies, parasitized RBC from mice with peak parasitemia were harvested and labelled with ^{51}Cr as previously described (16). Mice were injected intravenously with 10^8 ^{51}Cr-labelled PRBC in 0.2 ml of saline and bled at various time points from the retro-orbital plexus with a calibrated 25 µl micropipette. Spleens and livers were dissected out at the end of the 4-h observation period. Blood and tissue samples were counted in a Beckman gamma counter. Clearance curves were drawn by using counts expressed as percentages of the maximum counts obtained at either 2 or 5 min.

RESULTS AND DISCUSSION

In order to assess the importance of severe anemia in the lethality of *P. chabaudi* AS infections in susceptible mice, infected A/J mice were transfused with washed syngeneic blood cells at the time of peak parasitemia (day 7 in these experiments) and on two consecutive days thereafter. As shown in Fig. 1, 10 of 10 (100%) untransfused A/J mice succumbed to infection by day 10 whereas only 1 of 10 (10%) transfused mice died as of day 21, when the experiment was terminated (chi-square test, $P = 0.0001$). The kinetics of parasitemia and changes in RBC counts in control, untransfused, and transfused A/J mice are shown in Fig. 2. As previously shown by our laboratory (18), A/J mice developed high levels of peak parasitemia, which occurred in this experiment on day 7 (Fig. 2A). Parasitemia decreased between day 8 and day 9, when the lowest RBC counts occurred (1.52×10^9 $\pm 0.2 \times 10^9$ RBC per ml [mean $\pm$ standard deviation]). During this period of severe anemia, infected mice lost weight, became lethargic and hypothermic, and died. As expected, blood
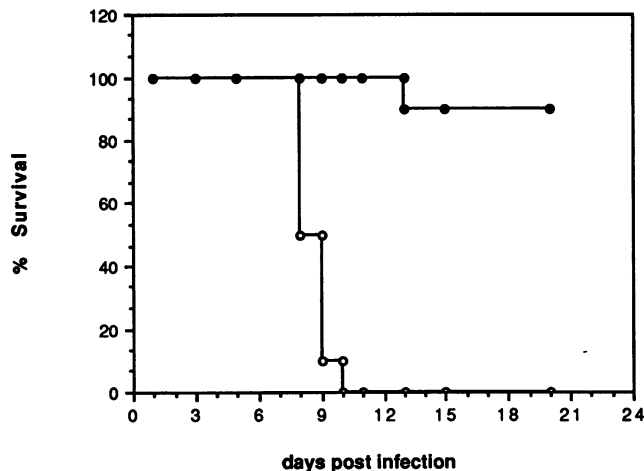
FIG. 1. Effect of blood transfusion on survival of susceptible A/J mice during *P. chabaudi* AS infection. Blood transfusions, performed as described in Materials and Methods, were given on days 7 to 9. Percent survival of untransfused control mice (open symbols [$n = 10$]) and transfused mice (closed symbols [$n = 10$]) is shown. Chi-square test, $P < 0.0001$.

transfusion of infected A/J mice on days 7 through 9 prevented the development of severe anemia (Fig. 2B). Unexpectedly, the parasitemia in transfused mice was maintained at patent levels (20 to 40%) for a period of 5 to 6 days after peak parasitemia. Parasitemia decreased to subpatent levels on day 14, 5 days after the last blood transfusion. Interestingly, the decrease in parasitemia was coincident with the development of moderate levels of anemia (RBC count, $3.05 \times 10^9 \pm 0.58 \times 10^9$/ml). These mice developed symptoms similar to, albeit less severe than, those of untransfused mice on days 8 to 9, but most of the animals survived and cleared the parasitemia to subpatent levels by day 15 postinfection. A single mouse died during this period. Transfused A/J mice rechallenged with 10^6 PRBC 30 days after primary infection did not develop patent parasitemia.

Thus, these observations indicate that anemia is a proximate cause of mortality in susceptible A/J mice and that susceptible mice are inherently capable of developing acquired immunity to *P. chabaudi* AS. The same conclusions were reached in an earlier study with a different rodent *Plasmodium* species (4); in that study, repeated blood transfusions rescued mice from an otherwise uniformly lethal *Plasmodium berghei* infection and allowed adequate time for the development of acquired immunity. However, unlike the present results, blood transfusion induced an early and steady decline in parasitemia in *P. berghei*-infected mice. Differences in RBC host preference between these two *Plasmodium* species may explain the divergence. Blood transfusion, consisting predominantly of normocytes, into *P. berghei*-infected mice may have inhibited endogenous erythropoiesis, thereby decreasing the number of reticulocytes, which are the preferred host cell of this species. On the other hand, blood transfusion into *P. chabaudi*-infected mice could conceivably provide the parasites with more target cells, as this species has been regarded as being predominantly normocytophilic (10).

We sought to confirm the phenomenon of transfusion-induced prolongation of patent parasitemia in resistant C57BL/6 mice infected with *P. chabaudi* AS. Infection in this mouse strain follows a self-limiting, nonlethal course and is
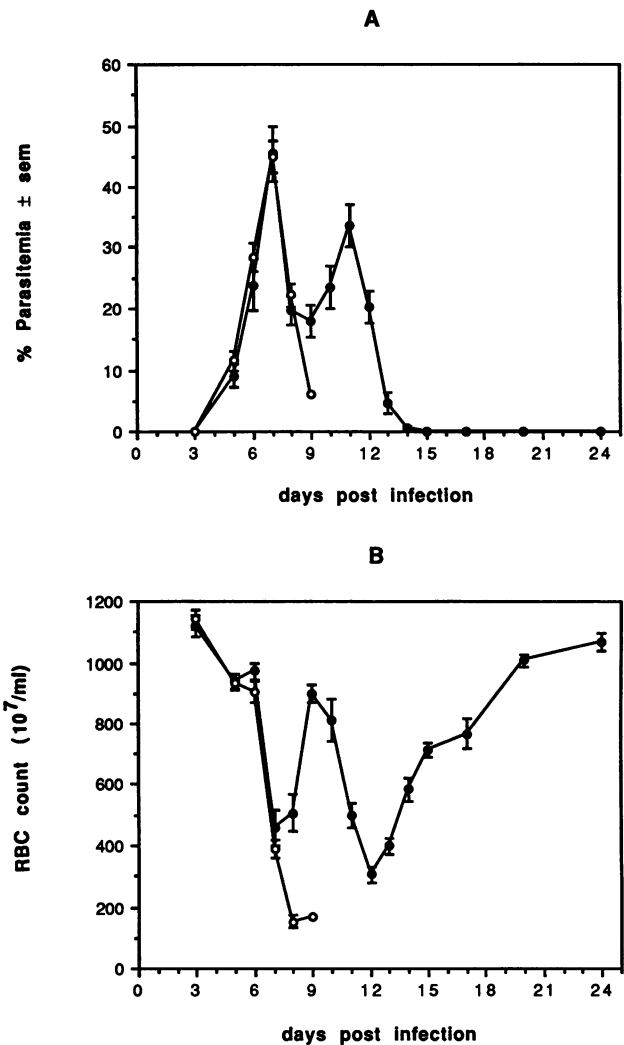

FIG. 2. Effect of blood transfusion on the course of *P. chabaudi* AS infection (A) and the course of anemia (B) in susceptible A/J mice. The percent parasitemia and RBC counts in untransfused control mice (open symbols) and in transfused mice (closed symbols) are shown. Values are means ± standard errors of the means (sem) for five mice per group. Data shown are representative of four independent experiments.

characterized by a primary patent parasitemia followed by a parasitologic crisis. One or more smaller recrudescences may occur before the infection is finally resolved by 4 weeks postinfection. As shown in Fig. 3, untransfused C57BL/6 mice experienced a progressive decrease in RBC counts reaching a low on days 9 to 10, at the time when crisis was occurring. Daily blood transfusion of infected C57BL/6 mice starting at peak parasitemia (day 7) up to day 11 maintained the RBC counts at normal or slightly subnormal levels and the parasitemia at patent levels (between 10 and 20%) for 6 days after peak parasitemia. As in the transfused A/J mice, the resolution of patent parasitemia (days 14 to 15) was coincident with the development of moderate levels of anemia. Waves of reticulocytosis followed the episodes of anemia in both untransfused and transfused animals, with peaks occurring on day 12 (57.1% ± 1.3% reticulocytes) and day 17 (42.2% ± 4.8% reticulocytes), respectively. RBC counts had approached normal levels
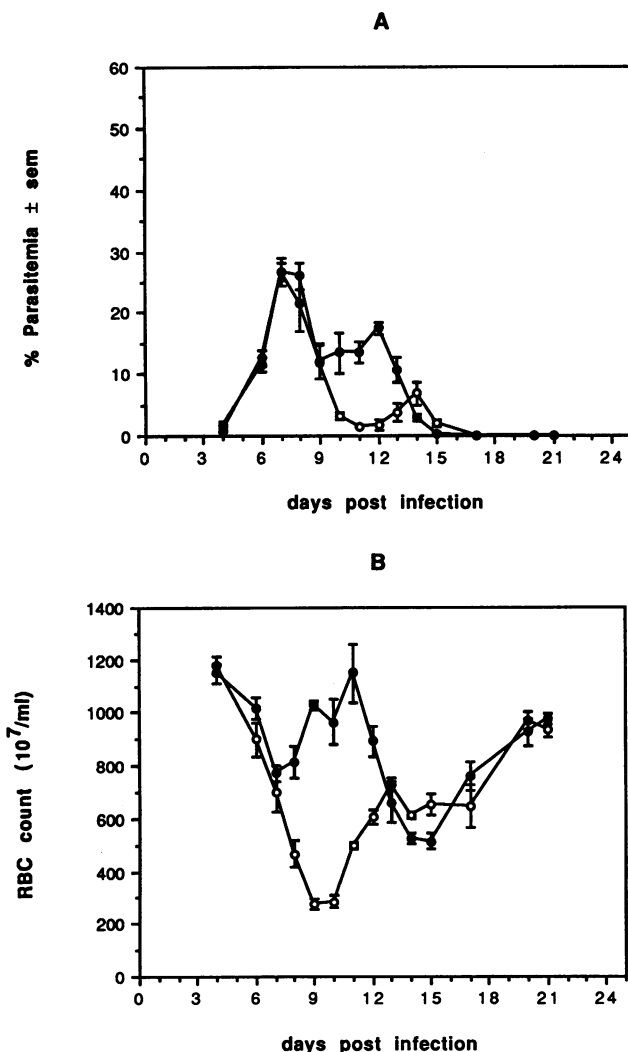
A

B

FIG. 3. Effect of blood transfusion on the course of *P. chabaudi* AS infection (A) and the course of anemia (B) in resistant C57BL/6 mice. The parasitemia and RBC counts in untransfused control mice (open symbols) and in transfused mice (closed symbols) are shown. Values are means ± standard errors of the means (sem) for four mice per group. This experiment was repeated twice with similar results.

by the end of the experiment. It is noteworthy that the rebound in RBC counts was transiently delayed in untransfused mice as a result of a secondary parasitemia, which peaked on day 14. Interestingly, no such secondary peak was observed in the transfused mice. Taken together, these observations demonstrate the modulation of parasitemia and timing of parasitologic crisis by infusion with syngeneic blood cells.

An obvious mechanism by which blood transfusion is able to modulate the course of infection is that RBC transfusion provides a pool of preferred target cells and prevents the release of reticulocytes, which are thought not to be the preferred target cell of this parasite species (10). This simple explanation seems untenable for two reasons. First, blood transfusion using preparations enriched for reticulocytes produced the same effect as the normocyte preparations (data not shown). Second, a recent report has indicated that the normocyte preference of *P. chabaudi* is more apparent than real (5).

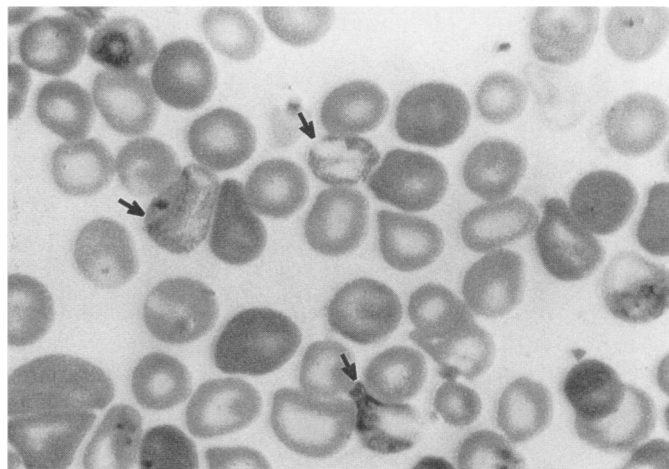In addition to its effects on course of infection, blood

FIG. 4. Blood smear from a representative, transfused C57BL/6 mouse, showing increased gametocyte differentiation. Arrows indicate gametocytes. Magnification, ×850.

transfusion increased the numbers of sexual-stage parasites (gametocytes) seen in the blood smears. This was most obvious in the blood of mice transfused with reticulocyte-rich preparations (Fig. 4). This observation is consistent with the results of recent in vitro studies with *Plasmodium falciparum*, which demonstrate that inclusion of either reticulocytes or hemolysates of infected RBCs in the cultures increased the propensity of the parasites to differentiate into gametocytes (15, 20). Both factors are likely to occur in the transfused mice and possibly favor sexual differentiation.

Experimental infection in animals with *Plasmodium* species is characterized by a prepatent phase of varying duration; a patent phase, when parasite growth rates are maximal; and a period of crisis, defined as a period of spontaneous attrition of the parasite population. During crisis, developmental degeneration and cytostasis of intraerythrocytic parasites occur. The process of crisis has been largely attributed to cell-mediated immune mechanisms (8). The cellular mechanisms which destroy intraerythrocytic parasites are thought to involve macrophages, lymphocytes, granulocytes, and factors derived from them (11). It is believed that activated T cells secrete lymphokines which activate effector cells to kill parasites by oxidative and nonoxidative mechanisms. These events are thought to occur in the microvascular space of the spleen, the liver, and the bone marrow. Based on ultrastructural studies of splenic tissue from *Plasmodium yoelii*-infected mice (21) and blood clearance and trafficking studies of physically damaged RBCs in *P. berghei*-infected rats (22), a model of how parasite destruction may occur in situ has been suggested (21). It has been proposed by Weiss and his colleagues (21) that patent infection is associated with the development of a splenic barrier system which effectively shunts blood circulation into the splenic sinuses. This results in a decreased capacity of the spleen to filter rheologically altered RBCs. During crisis, the splenic barrier system is "opened," with two interrelated consequences: entry of parasitized RBCs into the cordal circulation, allowing contact with activated effector cells, and the release of newly formed reticulocytes, which replenishes the peripheral RBC pool. Whether or not this paradigm applies universally to all *Plasmodium* species has not been determined. We reasoned that if this scenario occurred in *P. chabaudi*-infected mice, then blood transfusion would elimi-
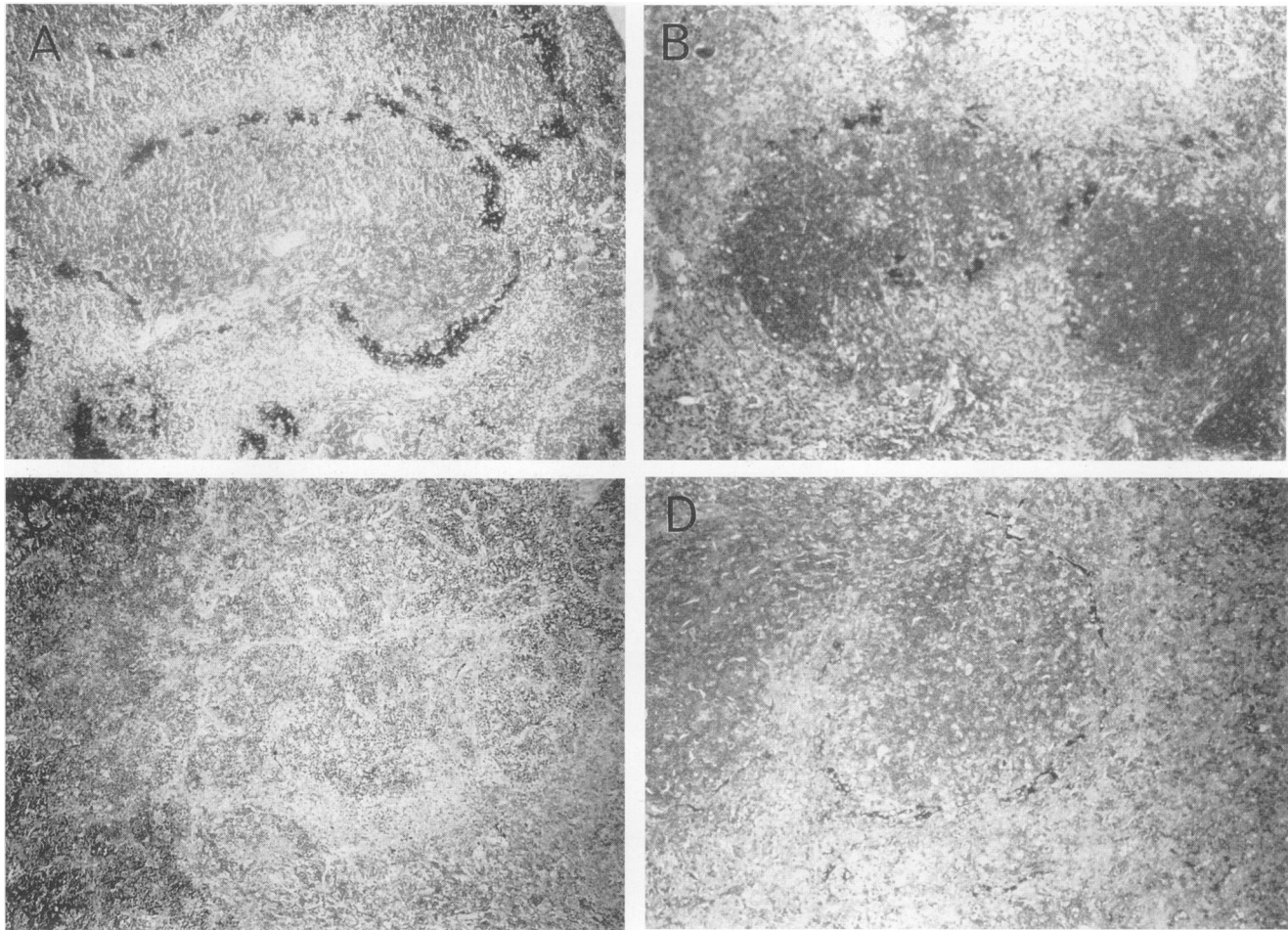
FIG. 5. Histology and carbon uptake in spleen sections obtained from a representative control C57BL/6 mouse (A), a mouse with peak parasitemia at day 7 (B), a mouse which was undergoing crisis at day 10 (C), and a transfused mouse also at day 10 (D). Magnification, ×120.

nate the physiologic trigger for the opening of the barrier systems required for the release of new reticulocytes, thereby preventing the close contact of PRBC with activated effector cells in the splenic cords. In order to provide evidence for or against our hypothesis, we performed histological and functional clearance experiments in normal mice, mice with peak parasitemia, mice undergoing crisis, and transfused mice. In contrast to the results with *P. berghei* and *P. yoelii*, we did not find evidence for an opening of splenic barriers at the time of crisis. In fact, infection was associated with a progressive decrease in clearance rate and splenic uptake of ^{51}Cr-labelled PRBC which was not reversed at the time of crisis (data not shown). In normal spleens (Fig. 5A), there is a sharp delineation of splenic red pulp and white pulp areas by a zone of carbon uptake by marginal zone macrophages. Carbon uptake in the marginal zone is diminished but still distinct in mice with peak parasitemia (Fig. 5B). In contrast, spleens of mice undergoing crisis revealed congestion with erythroblastic islands and a lack of carbon particle uptake in the marginal zone (Fig. 5C). These results suggest that crisis in murine *P. chabaudi* infections is not associated with the opening of barrier cell complexes. Furthermore, these findings, although by no means definitive, appear to be inconsistent with our hypothesis described above. However, direct ultrastructural analysis of spleens and in situ distribution studies of micro-

spheres would be required to resolve this question. It is interesting that the histologic features of spleens from transfused mice (Fig. 5D) resemble those of the spleens from mice with peak parasitemia rather than the spleens from mice at crisis. This probably reflects the similarity in parasitologic and hematologic features of the peripheral blood between transfused mice and mice with peak parasitemia.

The exact mechanism by which blood transfusion alters the course of parasitemia and the timing of parasite clearance is not known. We speculate that a shock-like syndrome associated with acute hemolysis and severe anemia initiates a cascade of systemic responses which may be detrimental to parasite growth and metabolism. Indeed, Rzepczyk and Clark (14) have previously shown that administration of endotoxin to mice infected with *Plasmodium vinckei* had a cytostatic effect on the parasite (14). It has been proposed that mediators released during systemic shock have deleterious or inhibitory effects on parasites. Mediators which have been implicated in the process of crisis during malaria include tumor necrosis factor (9), immune interferon (9), lipid peroxides (13), and crisis form factor (6). It is noteworthy that host tissues also suffer from anoxic and ischemic injury during the period of crisis. Indeed, Jensen (6) has previously noted that crisis is usually associated with severe infections, preceding either the death of the host or spontaneous resolution of parasitemia and

survival of the host. Our suggestion that the mechanism(s) which operates during crisis is mainly a nonspecific physiologic response rather than a specific immune-mediated process is consistent with experiments in mice with genetic or experimentally induced deficiencies in specific cell types or effector molecules. In murine hosts deficient in B cells and antibodies (anti-immunoglobulin M-treated mice [3] and severe combined immunodeficient [SCID] mice [7]), T cells (nude and SCID mice [7]; anti-CD4- and anti-CD8-treated mice [12]), and macrophages (silica-treated mice [17]) and in animals deficient in nitric oxide (N^Gmonomethyl-L-arginine-treated mice [19]) and oxidative metabolites (P/J mice [2]), there is a strikingly consistent decrease in levels of parasitemia during the period immediately following peak parasitemia. This decrease in parasitemia does not differ either in timing or in rate from that in control, immunocompetent mice. The essential difference between immunocompetent and immunodeficient hosts lies mainly in the phase after crisis, in which immunodeficient hosts experience a resurgence of patent parasitemia, which they are unable to suppress or sterilize. Thus, as previously suggested (1), different mechanisms likely operate to limit parasite multiplication, to induce and effect parasitologic crisis, to maintain the infection at subpatent levels, and to sterilize the infection.

The blood transfusion model described here allows the experimentalist a measure of control over the timing of crisis and the opportunity to discern which mechanisms, whether they be immune or physiological, contribute to this process. Furthermore, this model permits studies on the effects of prolonged exposure to high levels of parasites on the development of T-cell subsets and antibody responses and on the development of acquired immune responses in genetically susceptible mice.

In summary, our results clearly demonstrate that susceptible A/J mice succumb to infection as a result of severe anemia and other pathophysiologic alterations associated with it and that these mice are inherently capable of mounting an effective acquired immune response to P. chabaudi AS. Furthermore, we have presented novel observations which suggest that the process of crisis during blood-stage malaria may be regulated primarily by physiologic alterations associated with acute anemia. In addition, our observation of increased numbers of gametocytes in transfused mice suggests that the effect of blood transfusion on the transmission characteristics of the parasites (i.e., the infectivity to the mosquito vector) needs to be explored (9).

REFERENCES

1. Barnwell, J. H., and R. S. Desowitz. 1977. Studies on parasitic crisis in malaria. 1. Signs of impending crisis in Plasmodium berghei infection in the white rat. Ann. Trop. Med. Parasitol. 71:429–33.
2. Cavacini, L. A., M. Guidotti, L. A. Parke, J. Melancon-Kaplan, and W. P. Weidanz. 1989. Reassessment of the role of splenic leukocyte oxidative activity and macrophage activation in expression of immunity to malaria. Infect. Immun. 57:3677–3682.
3. Cavacini, L. A., L. A. Parke, and W. P. Weidanz. 1990. Resolution

4. Hejna, J. M., N. J. Rencricca, and R. M. Coleman. 1974. Effective recovery and immunity to virulent malaria following red cell transfusion at crisis. Proc. Soc. Exp. Biol. Med. 146:462–464.
5. Jarra, W., and K. N. Brown. 1989. Invasion of mature and immature erythrocytes of CBA/Ca mice by a cloned line of Plasmodium chabaudi chabaudi. Parasitology 99:157–163.
6. Jensen, J. 1989. Malaria crisis forms: intraerythrocytic development derangement, p. 109–126. In M. M. Stevenson (ed.), Malaria: host responses to infection. CRC Press, Inc., Boca Raton, Fla.
7. Meding, S. J., and J. Langhorne. 1991. CD4+ T cells and B cells are necessary for the transfer of protective immunity to Plasmodium chabaudi chabaudi. Eur. J. Immunol. 21:1433–1438.
8. Melancon-Kaplan, J., and W. P. Weidanz. 1989. Role of cell-mediated immunity in resistance to malaria, p. 37–63. In M. M. Stevenson (ed.), Malaria: host responses to infection. CRC Press, Inc., Boca Raton, Fla.
9. Naotunne, T. S., N. Karunaweera, G. Del Giudice, M. U. Kularatne, G. E. Grau, R. Carter, and K. N. Mendis. 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J. Exp. Med. 173:523–529.
10. Ott, K. J. 1968. Influence of reticulocytosis on the course of infection of Plasmodium chabaudi and P. berghei. J. Protozool. 15:365–369.
11. Playfair, J. H. L., K. R. Jones, and J. Taverne. 1989. Cell mediated immunity and its role in protection, p. 65–86. In M. M. Stevenson (ed.), Malaria: host responses to infection. CRC Press, Inc., Boca Raton, Fla.
12. Podoba, J. E., and M. M. Stevenson. 1991. CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage Plasmodium chabaudi AS. Infect. Immun. 59:51–58.
13. Rockett, K. A., G. A. T. Targett, and J. H. L. Playfair. 1988. Killing of blood-stage Plasmodium falciparum by lipid peroxides from tumor necrosis serum. Infect. Immun. 56:3180–3183.
14. Rzepczyk, C. M., and I. A. Clark. 1981. Demonstration of a lipopolysaccharide-induced cytostatic effect on malarial parasites. Infect. Immun. 33:343–347.
15. Schneweis, S., W. A. Maier, and H. M. Seitz. 1991. Haemolysis of infected erythrocytes—a trigger for the formation of Plasmodium falciparum gametocytes? Parasitol. Res. 77:458–460.
16. Smith, P. L., K. W. Hunter, E. C. Oldfield, and G. T. Strickland. 1982. Murine malaria: blood clearance and organ sequestration of Plasmodium yoelii-infected erythrocytes. Infect. Immun. 38:162–167.
17. Stevenson, M. M., E. Ghadirian, N. C. Phillips, D. Rae, and J. E. Podoba. 1989. Role of mononuclear phagocytes in elimination of Plasmodium chabaudi AS infection. Parasite Immunol. 11:529–544.
18. Stevenson, M. M., J. Lyanga, and E. Skamene. 1982. Murine malaria: genetic control of resistance to Plasmodium chabaudi. Infect. Immun. 38:80–88.
19. Taylor-Robinson, A. W., R. S. Phillips, A. Severn, S. Moncada, and F. Y. Liew. 1993. The role of TH1 and TH2 cells in a rodent malaria infection. Science 260:1931–1934.
20. Trager, W., and G. S. Gill. 1992. Enhanced gametocyte formation in young erythrocytes by Plasmodium falciparum in vitro. J. Protozool. 39:429–432.
21. Weiss, L., U. Geduldig, and W. P. Weidanz. 1986. Mechanisms of splenic control of murine malaria: reticular cell activation and the development of the blood-spleen barrier. Am. J. Anat. 176:251–285.
22. Wyler, D. J., T. C. Quinn, and L. T. Chen. 1981. Relationship of alterations in splenic clearance function and microcirculation to host defense in acute rodent malaria. J. Clin. Invest. 67:1400–1404.
23. Yap, G. S., and M. M. Stevenson. 1992. Plasmodium chabaudi AS: erythropoietic responses in resistant and susceptible mice. Exp. Parasitol. 75:340–352.